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AWARD NUMBER DAMD17-97-1-7036

TITLE: Restore Wild-Type Functions to P53 Mutants Using an RNA-Based Combinatorial Approach

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REPORT DATE: June 1998

TYPE OF REPORT: Annual

PREPARED FOR: Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. AGENCY USE ONLY *(Leave blank)*	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	June 1998	Annual (15 May 97 - 14 May 98)

4. TITLE AND SUBTITLE
Restore Wild-Type Functions to P53 Mutants Using an RNA-Based Combinatorial Approach

5. FUNDING NUMBERS
DAMD17-97-1-7036

6. AUTHOR(S)
Chen, Xiaoying, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
SRI International
Menlo Park, California 94025-3493

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 words)*

The tumor suppressor p53 plays pivotal roles in maintaining the integrity of the genome and regulates cell cycle arrest and programmed cell death (apoptosis). Tumors without a functioning p53 are more likely to have a very aggressive clinical course. We proposed to use an RNA-based combinatorial approach to select for single-stranded RNA molecules that will bind to a mutant p53 protein, correct its conformation, and restore its sequence-specific DNA-binding activity. We have successfully accomplished the tasks we proposed for the first year of funding, including (1) expression and purification of human wild-type p53 protein in insect cells, (2) *in vitro* selection of RNA aptamers from a pool of RNA molecules that bind to p53 protein, and (3) sequence-specific DNA binding assays using the EMSA technique. Sequence and structural analysis of the selected RNAs revealed that the ability of RNA molecules to interfere with sequence-specific DNA binding of p53 may depend on their tertiary structures.

14. SUBJECT TERMS
Breast Cancer
p53, RNA, SELEX, *In Vitro* Evolution, Tertiary Structure

15. NUMBER OF PAGES
17

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

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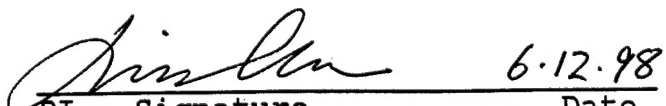

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INTRODUCTION

The tumor suppressor p53 plays pivotal roles in maintaining the integrity of the genome and regulates cell cycle arrest and programmed cell death (apoptosis) (Levine, 1997; Ko and Prives, 1996). p53 inactivation occurs more often in advanced breast cancer, and since loss of p53 functions results in increased cell proliferation and decreased cell death, tumors without a functioning p53 are more likely to have a very aggressive clinical course (Elledge et al., 1993). Alteration of a single amino in the p53 protein can drastically change its function—from a potent tumor suppressor to an oncogene (Milner, 1994). Thus, the development of novel therapeutics to restore wild-type function to mutant p53 proteins would be an innovative and highly specific strategy for treating human cancer.

Fast-accumulating *in vitro* and *in vivo* evidence shows that wild-type p53 protein and some mutants of p53 encoded by common "hot spot" alleles are not irreversibly defective in their DNA-binding ability but instead reside in a latent state due to protein conformational changes and can be activated to bind specific DNA sequences (Hupp and Lane, 1994). In particular, the cryptic DNA-binding function of mutant p53 proteins can be unmasked by molecular recognition between biomolecules such as short peptides, monoclonal antibodies, protein kinases, and single-stranded DNAs (Hupp et al., 1995; Abarzua et al., 1997; Hupp et al., 1992; Jayaraman and Prives, 1995).

We proposed to use an RNA-based combinatorial approach to select for single-stranded RNA molecules that will bind to a mutant p53 protein, correct its conformation, and restore its sequence-specific DNA-binding activity (Figure 1). This novel approach allows a fast, efficient search for functional nucleic acids that can potentially be developed for human gene therapy. Single-stranded RNAs and DNAs have a huge distribution of sizes and shapes and can not only function as enzymes (ribozymes) but also bind to proteins and small molecules tightly and specifically (Gold, 1995). We are using *in vitro* selection, or SELEX (Systematic Evolution of Ligands by EXponential enrichment), a powerful method that can find short RNA or DNA sequences with enzymatic functions or with tight binding affinity to a variety of targets (Fitzwater and Polisky, 1996; Conrad et al., 1996; Tuerk and Gold, 1990), to exploit the sequence and structure space of short oligonucleotides to build a repertoire of RNA molecules for recognizing and binding structural determinants of proteins such as p53, just as antibodies recognize and bind epitopes.

Our objectives for Year 1 were divided into three tasks, all of which have been successfully accomplished:

Task 1. Expression and purification of human wild-type p53 protein in insect cells.

Task 2. *In vitro* selection of RNA aptamers that bind to human wild-type p53 protein.

Task 3. Sequence-specific DNA binding assay to determine the effect of the selected RNA aptamers on the latent DNA-binding function of wild-type p53.

The work involved the following studies.

BODY: PROGRESS IN YEAR 1

Expression, Purification, and Characterization of Human Wild-Type p53 Protein.

The human wild-type p53 (hWTp53) gene (~1.3 kb) was inserted into an expression cassette (pCMH6K) downstream of a 5'GCCATGG-3' translation initiation sequence and a sequence encoding amino acids MA-YPYDVPDUYA (the hemaglutinin epitope)-R-HHHHHH for Ni-affinity chromatography (Wang et al., 1993). The resulting sequence flanked by unique *Nhe*I and *BamH*I sites was subcloned into a baculovirus expression vector (pIT) for infecting insect cells. Sf9 insect cells (Invitrogen, San Diego, CA) were infected with high titer recombinant baculovirus carrying hWTp53 (provided by Prof. Peter Tegtmeyer at the State University of New York at Stony Brook). Large scale expression was carried out in three T150 tissue culture flasks. The cells were harvested and lysed in buffer containing 150 mM Tris-HCl at pH 9.0, 150 mM NaCl , 0.5% NP-40, 10% glycerol, 2 μM PMSF, 50 μg/ml aprotinin, 50 μg/ml leupeptin, 10 μg/ml pepstatin A, 2 mM benzamidine, and 1 mM β-mercaptoethanol. Cell lysates were passed through a Ni-NTA agarose column (Qiagen, Inc., Valencia, CA) and washed with buffers suggested by the manufacturer. The proteins were dialyzed against buffer containing 20 mM Tris-HCl at pH 8.0, 100 mM NaCl, and 50% glycerol overnight at 4 °C and stored in aliquots of 1 mg/ml at -80°C. The purity of hWTp53 was assessed by sodium dodecyl sulfate (SDS) gel electrophoresis and gel staining with Coomassie blue.

To verify that the purified p53 protein retains its specific DNA binding activity, we used an electrophoretic mobility shift assay (EMSA) to monitor the binding of p53 on its consensus DNA sequence (Wang et al., 1993). The results (Figure 2) demonstrated that the purified human recombinant p53 has wild-type functions, i.e., binding specifically to its consensus DNA site (Funk et al., 1992) and being specifically recognized by its antibodies, PAb 421 and PAb 1801 (Hupp and Lane, 1992). In control experiments, nonspecific competitor DNA did not bind to p53, and non-p53-specific antibody (a monoclonal antibody against cdc2) could not induce a supershift of the DNA/p53 complex on the gel.

The consensus p53 DNA-binding site and the nonspecific competitor have the following sequences:

Consensus:	5'-GGACATGCCCGGGCATGTCC-3'
Competitor:	5'-TTGTTTTTGGCAGTGTGCCAACAGTCG-3'

Construction of the RNA Library

We synthesized a large pool of DNA templates containing an insert that was a completely randomized 40-nucleotide-long sequence flanked by defined sequences, including a 3 ' -PCR primer and a 5'- PCR primer containing the promoter for T7 RNA polymerase (Figure 3). Because of limitations in the amount of starting material and uneven coupling efficiencies in DNA synthesis, the number of theoretically possible sequences ($4^{40} \sim 10^{24}$ different molecules) exceeds the actual number of molecules (~10^{14}) used at the outset of the experiment (Fitzwater and Polisky, 1996). Double-stranded DNA molecules were amplified by PCR, purified on a 8%

polyacrylamide gel, and transcribed to yield the initial pool of 75-nucleotide RNAs by using a T7 RNA polymerase kit (Epicentre Technologies, Madision, WI). .

In vitro Selection of RNA Aptamers that Bind to hWTp53 Protein

We used a nitrocellulose filter binding assay to select RNA aptamers that can bind to a protein target with high affinity (Gold et al., 1995; Conrad et al., 1996; Keen, 1996). The initial pool of RNA (20 μg) in 200 μl of 1XBB (binding buffer) containing 25 mM HEPES at pH 7.4, 50 mM KCl, 1 mM DTT, 1 mM $MgCl_2$, 0.2 mM EDTA, 10% glycerol, and 0.5 mM PMSF (Tian et al., 1995) was heated to 94°C for 3 min. and then cooled to 4°C. Prior to the selection, the folded RNA pool was passed through a modified cellulose filter (e.g., a HAWP filter, Millipore Corp.) every other cycle to remove potential filter-binding sequences. The protein target, hWTp53 protein (1 μg), was mixed with the initial pool of RNA in 200 μl of 1XBB buffer and incubated at ambient temperature for 1 h. The mixture was passed through a HAWP filter held by a filter holder (13 mm, Corning Costar Co., Cambridge, MA) on a vacuum manifold (J. T. Baker, Phillipsburg, NJ) under a pressure of 5 inches of mercury (Conrad et al., 1996). The filter was washed twice with 200 μl of 1 X BB and the RNA:protein complexes retained by the filter were subsequently isolated. The RNA bound to p53 was eluted from the filter by mixing with 200 μl of 7 M urea and extracted with 600 μl of phenol-chloroform at pH 8.0. The eluted RNA was ethanol-precipitated in presence of 5 μg glycogen and redissolved in 20 μl of RNase-free water. Initially, the complex population of RNA variants contained a very low concentration of molecules with the desired activity (Tuerk and Gold, 1990). The selected RNAs were transcribed to cDNAs by avian myeloblastosis virus reverse transcriptase (AMV-RT) in a total volume of 50 μl on a thermocycler following the scheme: denaturation at 75°C for 3 min, renaturation at 25°C for 5 min, addition of 20 units of AMV-RT, and incubation at 37°C for 30 min and then at 48°C for 15 min (Fitzwater and Polisky, 1996). The cDNA was amplified by PCR (total reaction volume: 2 × 100 μl) as follows: denaturation at 94°C for 3 min, 25 cycles (94°C/45 s, 55°C/45 s, and 72 °C/1 min), further extention at 72°C for 7 min. The pool of RNA for next cycle of selection was generated by using the Epicentre T7 RNA polymerase kit. The entire SELEX process was repeated for 10 cycles of selection. This process is expected to yield progressively smaller and simpler populations with a greater mean binding affinity after a minimum of 10 cycles of selection and amplification.

Individual sequences from the active populations were isolated using cDNA cloning by using the Original TA Cloning kit (Invitrogen, San Diego, CA) to sample the composition of the RNA population. Eighteen colonies that showed positive responses to the X-gal treatment were picked and subcultured to yield plasmids, which were purified by using QIAQUICK minispin columns (Qiagen, Valencia, CA). The cloned DNA fragment was PCR amplified by using the SELEX 5'- and 3'- primers (Fig. 3), and the plasmid as a template. The RNA was then transcribed from each individual DNA fragment by using the Epicentre T7 RNA polymerase kit.

Analysis of RNAs Selected from the SELEX Procedures

Fourteen of the plasmids discussed above were sequenced by an automatic DNA sequencer, and six of them contained the insert. The RNA sequences for each of the six inserts are listed in Figure 4.

The secondary structures and $\Delta G°$ (37°C) values (thermodynamic stabilities) for these RNAs were predicted by using the program MulFold (Jaeger et al., 1989), and the results are shown in figure 5.

Interaction of RNA Molecules with hWTp53 Protein

We further studied the effect of RNA molecules selected by SELEX on the sequence-specific DNA binding of hWTp53 protein. RNAs transcribed from clones #3, 6, 9, 12, 15, 17 at 50 ng/μl were renatured in a buffer containing 25 mM HEPES at pH 7.4 and 50 mM KCl by heating at 94°C for 3 min and cooling at 4°C on a thermocycler. The sequence-specific DNA binding activity of hWTp53 protein in the presence of $tRNA^{phe}$ or selected RNAs was studied by EMSA following the procedures described by Wang et al., 1993. Figures 6 and 7 show that the presence of the selected RNA molecules or $tRNA^{phe}$ inhibits the sequence-specific DNA binding activity of p53.

Recently, it was reported that binding of large but not small DNAs or RNAs including $tRNA^{phe}$ by the C-terminus of p53 negatively regulates sequence-specific DNA binding by the central domain (Anderson et al., 1997). Our results demonstrated that all of the RNAs interfere with the sequence-specific DNA binding activity of p53, but there is a wide difference in the extent of inhibition by different clones of the selected RNAs. Most strikingly, RNA #12, followed by $tRNA^{phe}$, demonstrated the least interference with the sequence-specific DNA binding of p53. However, there is no correlation between activity of inhibition and thermo-dynamic stability, as determined by comparing the predicted $\Delta G°$ (37°C) values for each of the selected RNA molecules. Unlike other clones of selected RNA, RNA #12 folds into a secondary structure with three stems of approximately equal length (Fig. 5). It is tempting to speculate that RNA#12 may fold into a more compact tertiary structure, similar to that of the hammerhead RNA ribozyme with a similar secondary structure (Feig et al., 1998). Thus, compared with other selected RNAs with bulkier or more elongated tertiary structure, RNA#12 may exert less interference with sequence-specific DNA binding by the central domain. Alternatively, RNA#12 may bind with domains of p53 other than the C-terminus and thus cause less interference. The binding affinities of these RNA to the p53 structural domains are currently under investigation.

CONCLUSIONS

We have successfully accomplished the tasks we proposed for the first year of funding. These tasks include (1) expression and purification of human wildtype p53 protein in insect cells, (2) *in vitro* selection of RNA aptamers from a pool of RNA molecules with randomized

sequences of 40 nucleotides, and (3) sequence-specific DNA binding assays using the EMSA technique.

We have established the methodology for *in vitro* selection of RNA molecules binding to p53 protein by using a nitrocellulose filter binding assay, cloning of selected sequences into bacterial vectors, and sequence analysis of each individual clone of selected aptamers. There is no obvious consensus in the primary sequences of the selected RNA molecules. Computer-aided calculation of secondary structures of the selected RNAs revealed that all of the RNAs fold into stem-loop structures with thermodynamic stability higher than -10 kcal/mol. All of the selected RNAs inhibited the sequence-specific DNA binding activity of p53, but RNA #12 showed exceptionally low interference. Since RNA #12 has three long stems, we hypothesize that it may adopt a much more compact tertiary structure than other selected RNAs with possibly more elongated tertiary structures.

Our results show for the first time that, contrary to the published literature, relatively small RNAs (75-mers) inhibit the sequence-specific DNA binding activity of p53, and the extent of inhibition may depend on the tertiary folding of the RNA molecules. Further in-depth analysis of the tertiary structures of the selected RNA and binding affinity assays will shed light on this issue.

For the next year of funding we plan to conduct our research following Technical Objective 2 outlined in Statement of Work in our proposal "*In Vitro* Selection of RNA Aptamers that Bind to Mutant p53 Protein (p53His175) and Restore Its Squence-Specific DNA Binding Function."

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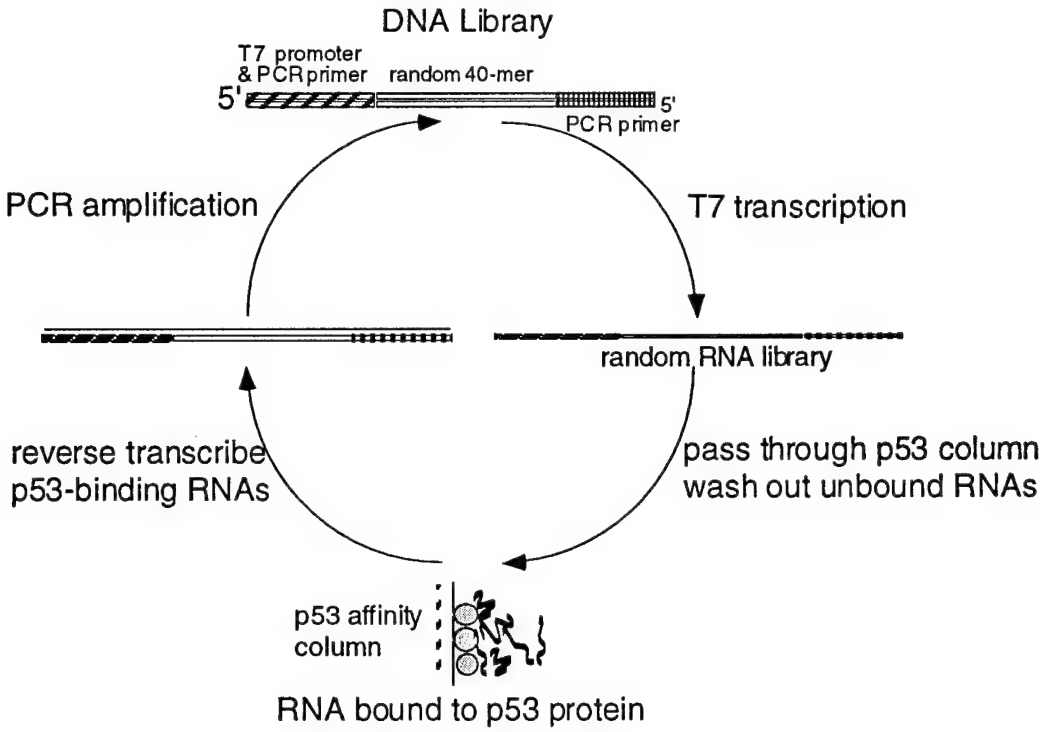


Figure 1. *In Vitro* Selection of Functional RNA Molecules from a Pool of Variants. A population of RNA sequence variants is generated by *in vitro* transcription of DNA templates that have been randomized during solid state chemical synthesis. RNA molecules are selected based on binding affinity to the p53 protein. Molecules that specifically bind to an immobilized p53 are eluted and reverse transcribed to yield a subpopulation of DNA templates, which will be amplified by PCR. The same round of selection will be repeated until the desired activity of RNA molecules is achieved.

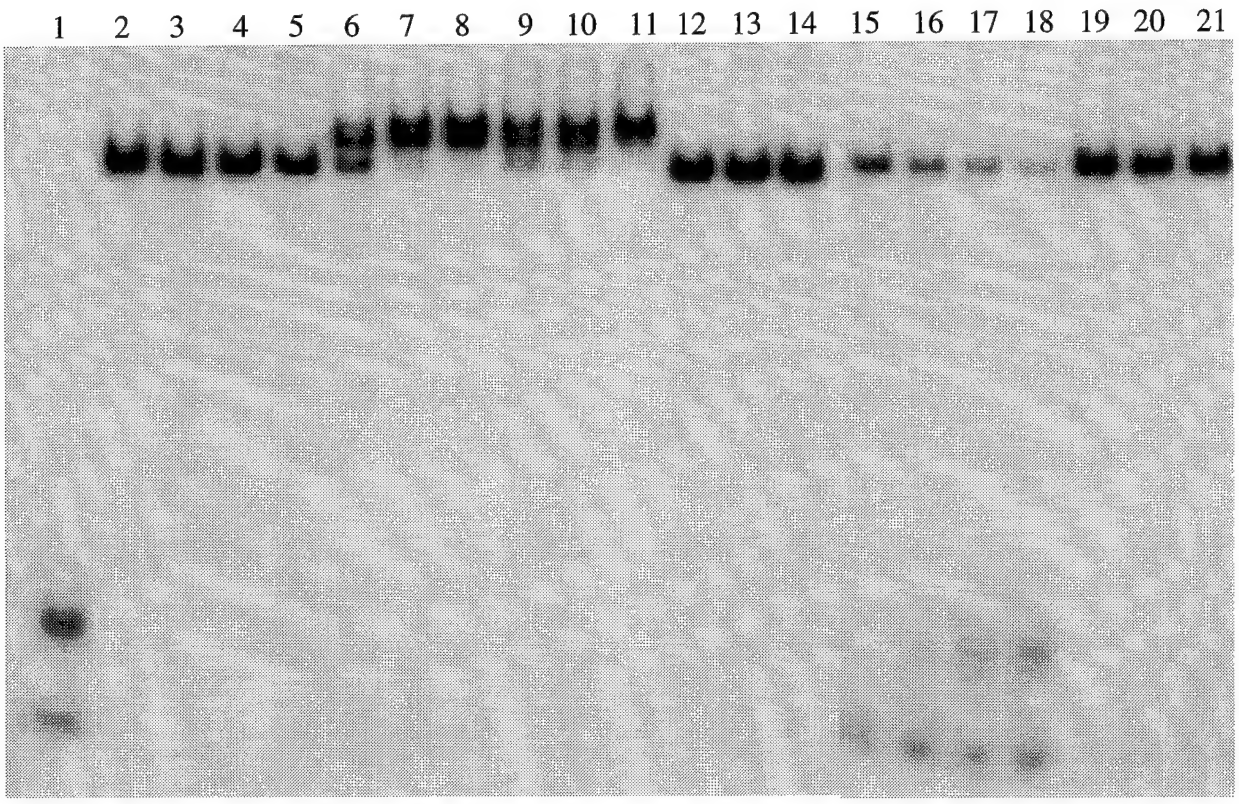


Figure 2. EMSA of the purified human wild-type p53 protein using the ^{32}P-labeled consensus p53-binding DNA probe; Lane 1: in the absence of p53 protein; Lanes 2-5, in the presence of 1, 0.5, 0.25, and 0.1 μg of p53, respectively; Lanes 6-8, in the presence of 0.5 μg p53 and 0.1, 0.2 and 0.3 μg of p53-specific antibody PAb421, respectively; Lanes 9-11, in the presence of 0.5 μg p53 and 0.1, 0.2 and 0.3 μg of p53-specific antibody PAb 1801, respectively; Lanes 12-14, in the presence of 0.5 μg p53 and 0.1, 0.2 and 0.3 μg of non-specific antibody against cdc2, respectively; Lanes 15-18, in the presence of 0.5 μg p53 and competed with 0.02, 0.05, 0.1 and 0.2 μg of the consensus p53-binding DNA, respectively; Lanes 19-21, in the presence of 0.5 μg p53 and competed with 0.02, 0.05, and 0.1 μg of the non-specific DNA, respectively.

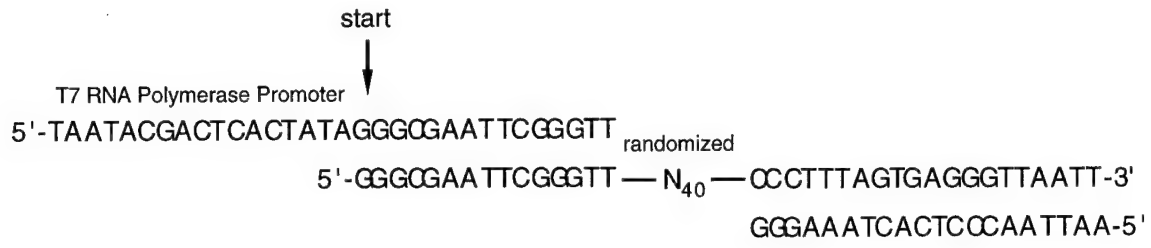


Figure 3. Sequences of 5′ and 3′ PCR primers and the template for SELEX. The DNA oligomers were made on an Applied Biosystem 380B DNA synthesizer. The stretch of 40 random bases were generated by adding equal amounts of each phosphoramidite at each position. The DNAs containing the random sequence were amplified using the other two sequences as PCR primers. The resulting double-stranded DNA incorporated a T7 polymerase promoter that was used as a template for the production of single-stranded RNAs. The transcription site is marked.

RNA#3
GGGCGAAUUCGGGUUUGGUAUGCGGGGUUACUAAUUAGUCGAGUGUGUUGUUUCUCCCUUUAGUGAGGGUUAAUU

RNA#6
GGGCGAAUUCGGGUUAUGGUGGUACUGAUGGUGGUUCUCGUUGCAGUCCACGUGUCCCUUUAGUGAGGGUUAAUU

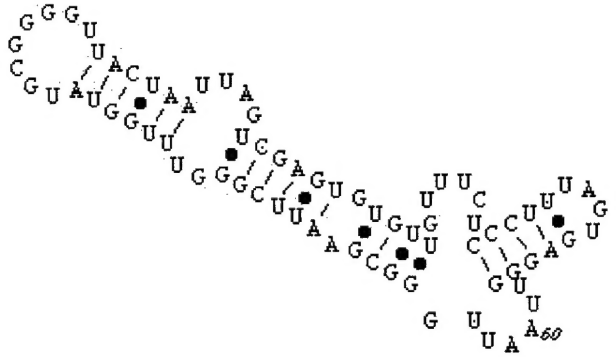
RNA#9
GGGCGAAUUCGGGUUUUUGGUAGUGGAGGUAGGUACUGUGGUUUUGUCCGUCGGUCCCUUUAGUGAGGGUUAAUU

RNA#12
GGGCGAAUUCGGGUUGGAUAGUAGGCGCAUAUGGCAUCUUCGUGGUUGUGUAUUGCCCUUUAGUGAGGGUUAAUU

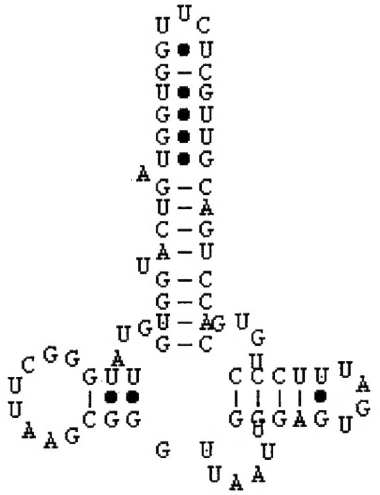
RNA#15
GGGCGAAUUCGGGUUUUUAGGGAGGGACUAUUUUAAGAGAGUCGCCGUCGGUGUCCCCUUUAGUGAGGGUUAAUU

RNA#17
GGGCGAAUUCGGGUUUUGUAGUGGUGGAGGAGGUUCUCGGUUCGUGCGUGGCUAGCCCUUUAGUGAGGGUUAAUU

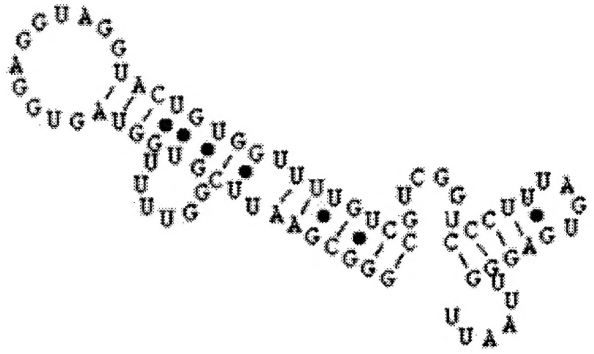
Figure 4. Sequences of selected RNAs.



RNA#3 (E = -11.3 kcal/mol)



RNA#6 (E = -14.7 kcal/mol)



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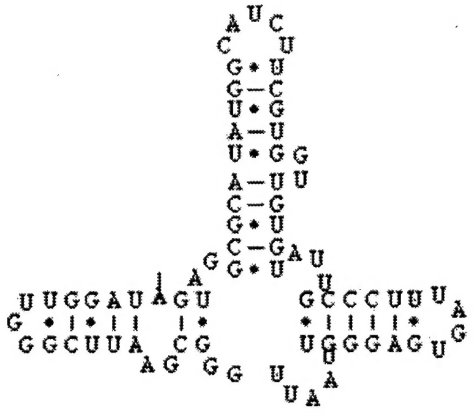
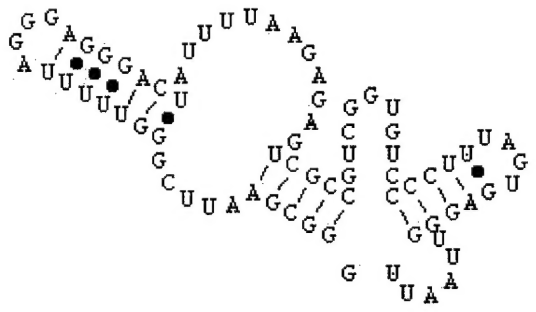


RNA#12 (E = -12.6 kcal/mol)



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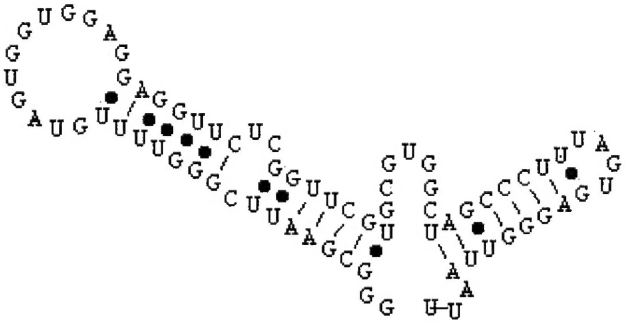

RNA#17 (E = -14.3 kcal/mol)

Figure 5. Predicted secondary structures and $\Delta G°(37°C)$ of selected RNAs.

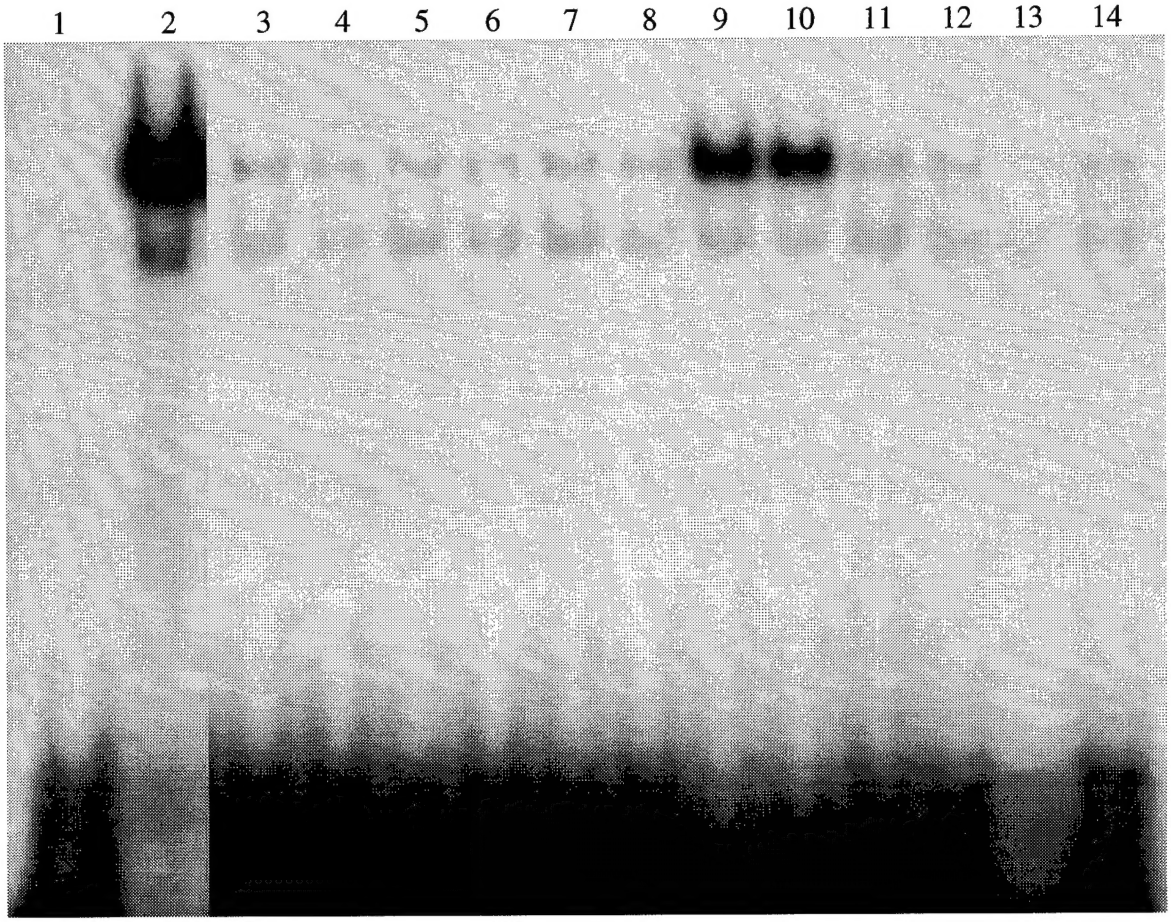

Figure 6. The Effect of selected RNAs on the sequence-specific binding activitity of human wild-type p53 protein. ^{32}P-labeled consensus p53-binding DNA probe was used and the EMSA was run under the same conditions as described in the legend of Fig. 2; Lane 1, in the absence of p53 protein; Lanes 2, in the presence of 1μg of p53; Lanes 3-14, with 0.3 μg of p53. Lanes 3 and 4, in the presence of 0.05 and 0.1 μg of RNA#3, respectively; Lanes 5 and 6, in the presence of 0.05 and 0.1 μg of RNA#6, respectively; Lanes 7 and 8, in the presence of 0.05 and 0.1 μg of RNA#9, respectively; Lanes 9 and 10, in the presence of 0.05 and 0.1 μg of RNA#12, respectively; Lanes 11 and 12, in the presence of 0.05 and 0.1 μg of RNA#15, respectively; Lanes 13 and 14, in the presence of 0.05 and 0.1 μg of RNA#17, respectively.

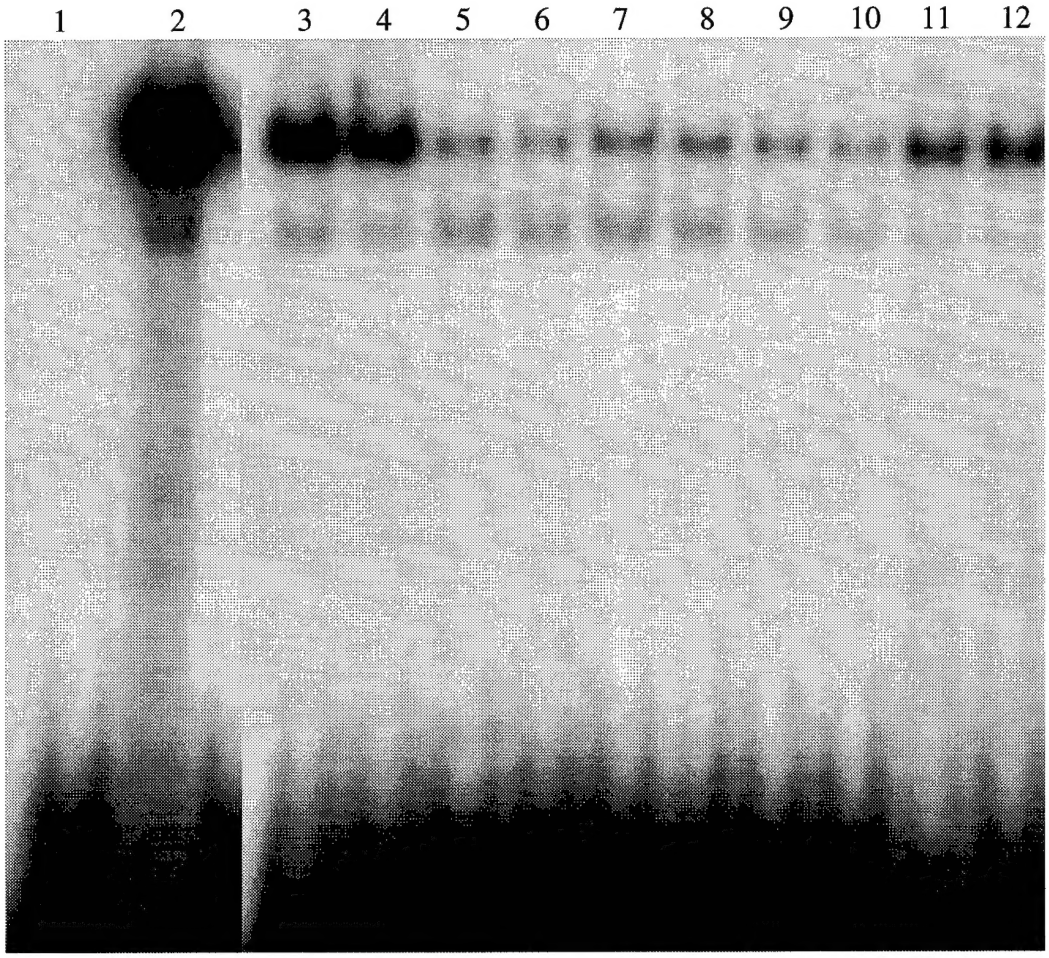

Figure 7. The Effect of selected RNAs and $tRNA^{phe}$ on the sequence-specific binding activitity of human wild-type p53 protein. ^{32}P-labeled consensus p53-binding DNA probe was used and the EMSA was run under the same conditions as described in the legend of Fig. 2. Lane 1: in the absence of p53 protein; Lanes 2, in the presence of 0.2 μg of p53; Lanes 3-12, in the presence of 0.2 μg of p53. Lanes 3 and 4, in the presence of 0.1 and 0.2 μg of RNA#12, respectively; Lanes 5 and 6, in the presence of 0.1 and 0.2 μg of RNA#17, respectively; Lanes 7 and 8, in the presence of 0.1 and 0.2 μg of RNA#15, respectively; Lanes 9 and 10, in the presence of 0.1 and 0.2 μg of RNA#3, respectively; Lanes 11 and 12, in the presence of 0.1 and 0.2 μg of $tRNA^{phe}$, respectively.